Dietary Folate and APC Mutations in Sporadic Colorectal Cancer.

Dietary Folate and *APC* Mutations in Sporadic Colorectal Cancer[1]

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Abstract

Folate deficiency has been associated with colorectal cancer risk and may be involved in colorectal carcinogenesis through increased chromosome instability, gene mutations, and aberrant DNA methylation. Within the Netherlands Cohort Study on diet and cancer, we investigated the associations between dietary folate intake and colorectal cancer risk with (APC^+) and without (APC^-) truncating *APC* mutations, accounting for hMLH1 expression and *K-ras* mutations. In total, 528 cases and 4200 subcohort members were available for data analyses of the study cohort ($n = 120{,}852$) from a follow-up period between 2.3 and 7.3 y after baseline. Adjusted gender-specific incidence rate ratios (RR) over tertiles of folate intake were calculated in case-cohort analyses for colon and rectal cancer. Although relatively high folate intake was not associated with overall colorectal cancer risk, it reduced the risk of APC^-colon tumors in men (RR 0.58, 95% CI 0.32–1.05, $P_{trend} = 0.06$ for the highest vs. lowest tertile of folate intake). In contrast, it was positively associated with APC^+ colon tumors in men (highest vs. lowest tertile: RR 2.77, 95% CI 1.29–5.95, $P_{trend} = 0.008$) and was even stronger when the lack of hMLH1 expression and *K-ras* mutations were excluded (RR 3.99, 95% CI 1.43–11.14, $P_{trend} = 0.007$). Such positive associations were not observed among women; nor was folate intake associated with rectal cancer when *APC* mutation status was taken into account. Relatively high folate consumption reduced the risk of APC^- colon tumors, but folate intake was positively associated with APC^+ colon tumors among men. These opposite results may indicate that folate enhances colorectal carcinogenesis through a distinct *APC* mutated pathway. J. Nutr. 136: 3015–3021, 2006.

Introduction

Folate deficiency is hypothesized to increase the risk of colorectal carcinogenesis through various mechanisms (1). It can cause uracil misincorporation in DNA, which in turn may lead to DNA strand breaks and chromosome instability. In addition, a low folate status can cause global DNA hypomethylation and hypermethylation of promoter regions, which may impair the expression of tumor suppressor genes and DNA repair genes (1). Considering these mechanisms, it could be expected that sufficient intake of dietary folate protects against colorectal cancer. Epidemiological studies on the relation between dietary folate intake and colorectal cancer risk however, have not consistently shown a protective effect of high folate intake (2,3). This inconsistency may partly be explained by the possible dual role of folate in carcinogenesis; that is, folate would prevent carcinogenesis in normal healthy tissue but may promote growth of existing tumors (4). In addition, accounting for molecular events underlying the carcinogenic process may further clarify the effect of folate intake on colorectal carcinogenesis, but this was not addressed in most of these epidemiological studies.

A well-characterized molecular alteration in colorectal carcinogenesis is the occurrence of mutations in the adenomatous polyposis coli (*APC*)[7] gene. Somatic mutations in this tumor suppressor gene were found mainly in its mutation cluster region in a significant proportion of sporadic colorectal carcinomas, varying from 34 to >80% (5–8). This underscores the importance of *APC* gene mutations in sporadic colorectal carcinogenesis. In an earlier study in colorectal cancer patients (8) we found that 37% of the patients had tumors harboring an *APC* mutation resulting in a stop codon, which leads to a truncated and therefore inactive *APC* protein. In addition, Diergaarde et al. (9) found an indication for a positive association between folate intake and

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[7] Abbreviations used: APC, adenomatous polyposis coli; hMLH1, human mut-L homologue 1; K-ras, Kirsten ras; MSI, microsatellite instability; NLCS, Netherlands Cohort Study; O^6-MGMT, O^6-methylguanine DNA methyltransferase; PALGA, Pathologisch Anatomisch Landelijk Geautomatiseerd Archief; RR, incidence rate ratio.

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colorectal adenomas, but the associations did not differ between adenomas with or without truncating *APC* mutations. In another study, Diergaarde et al. (10) observed a differential association between the consumption of green leafy vegetables, an important source of dietary folate, and the risk of carcinomas with or without truncating *APC* mutations.

The exact mechanism through which folate may alter *APC* functionality remains unclear, however. Possibly, there is an indirect effect of decreased activity of the DNA repair gene O^6-methylguanine DNA methyltransferase (*O^6-MGMT*), which was found to be associated with hypermethylation of its promoter region (11). Consequently, this can lead to an increased risk of tumors with G:C > A:T mutations in other key genes, like *APC*, involved in colorectal cancer (12,13).

Several studies have suggested that colorectal tumors may arise through distinct pathways based on specific molecular alterations. In this respect, mutations in *APC* and Kirsten ras (*K-ras*) occur less often in tumors with microsatellite instability (MSI) compared with microsatellite stable ones (14–17). MSI is known to be strongly associated with the lack of expression of the DNA mismatch repair gene human mut-L homologue 1 (*hMLH1*) (18,19). Tumors harboring this type of instability occur predominantly in the proximal colon, are often poorly differentiated, and are more frequently present in women at an older age (14,20). Furthermore, exogenous factors such as smoking behavior (21,22) and dietary factors (7,23–25) may be differentially associated with the risk of cancer development in MSI and non-MSI tumors. In addition, differential associations were also observed between folate intake and mutations in *K-ras* (26–29). Therefore, it is important to account for potential aberrations in *K-ras* and *hMLH1* and for differences in tumor location and sex when assessing the potential effect of dietary folate on colorectal cancer risk and *APC* mutations.

In view of these findings, this study aimed to investigate the associations between folate intake and the risk of colon and rectal cancer, with and without truncating *APC* mutations. We also assessed whether these associations were independent of *K-ras* mutations and hMLH1 expression. The study was conducted within the framework of the Netherlands Cohort Study on diet and cancer (NLCS), of which the study population consumed relatively low levels of dietary folate (insofar as folic acid fortification is not allowed in the Netherlands) compared with some other Western countries.

Subjects and Methods

Study population. The participants in this study were incident colon and rectal cancer patients and subcohort members from the NLCS, which has been described in detail elsewhere (30). Briefly, the study was initiated in 1986 and includes 58,279 men and 62,573 women, aged 55–69 y at baseline, who originated from 204 Dutch municipalities with computerized population registers. Numbers of subcohort members and cases are displayed in a flow chart (**Fig. 1**). At baseline, participants completed a self-administered food frequency questionnaire that also contained questions about other risk factors for cancer. The entire cohort has been monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (9 cancer registries in The Netherlands) and to Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide network and registry of histopathology and cytopathology reports (31). Accumulation of person-time in the cohort was estimated through biennial vital status follow-up of a subcohort of 5000 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than nonmelanoma skin cancer were excluded from this subcohort, which left 4774 men and women eligible for analysis.

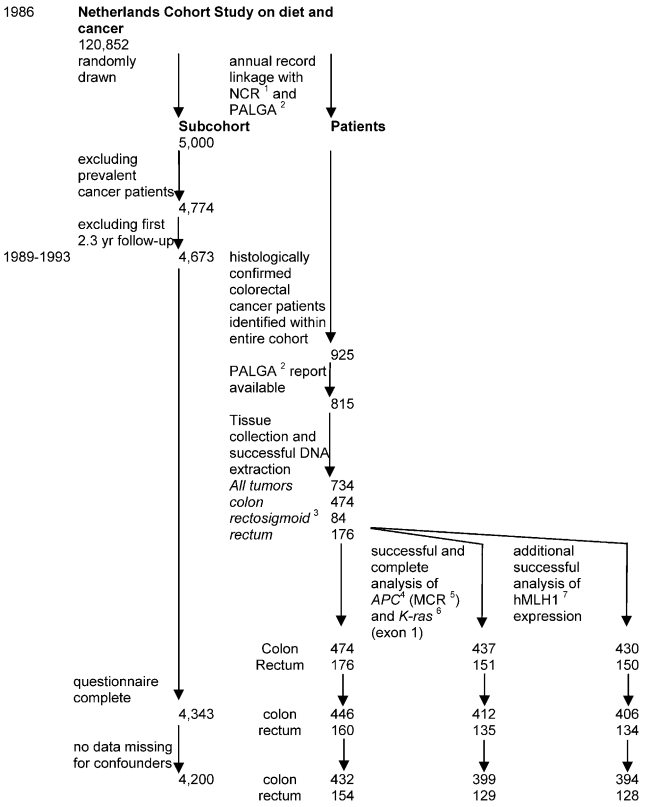

Figure 1 Flow diagram of subjects on whom the analyses were based
[1] Netherlands Cancer Registry.
[2] Pathologisch Anatomisch Landelijk Geautomatiseerd Archief.
[3] Patients with rectosigmoid tumors were not included in the analyses.
[4] Adenomatous polyposis coli.
[5] Mutation cluster region.
[6] Kirsten ras.
[7] human mut-L homologue 1.

The PALGA registry was not yet implemented in some of the municipalities included in the study in 1986 but reached full coverage by the end of 1988. Incomplete coverage can introduce selection bias, and preclinical disease can affect exposure status. For these reasons, we excluded the 1st 2.3 y of follow-up from the analyses. One hundred and one subcohort members were either deceased or diagnosed with cancer other than nonmelanoma skin cancer within this period, leaving 4673 men and women for analysis. From 1989 to 1994, 925 incident cases were identified with histologically confirmed colorectal cancer of which 815 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. Colorectal cancer was classified according to disease site as follows: colon, i.e., proximal colon (ICD-O-1 codes 153.0, 153.1, 153.4, 153.5, and 153.6), distal colon (153.2, 153.3, 153.7, 153.8, and 153.9), rectosigmoid (ICD-O-1 code 154.0), and rectum (ICD-O-1 code 154.1). Information about age, sex, and family history of colorectal cancer at baseline was retrieved from the NLCS database.

Tissue samples. Tumor material of the colorectal cancer patients was collected after approval by the ethical review boards of Maastricht University, the NCR, and PALGA (32). In addition, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available from PALGA upon request. From 815 tissue samples that were scattered over 54 pathology laboratories in the Netherlands, 734 samples (90%) could be traced and were retrieved between August 1999 and December 2001.

Gene mutation analyses. Genomic DNA was isolated from the paraffin sections after macrodissection of tumor cells as previously described (32). Gene mutation analyses of the mutation cluster region in

APC (codons 1286–1520), was performed as previously described (8). Briefly, nested PCR was used to amplify the mutation cluster region in 4 overlapping DNA fragments, and the purified fragments were sequenced. Two observers independently evaluated the sequence patterns and data entry. For 72 colorectal cancer patients, one or more fragments of the *APC* gene could not be analyzed completely, leaving 662 patients for data analysis.

The exon 1 fragment of the *K-ras* oncogene, spanning codons 8–29, was analyzed successfully for all 734 patients using nested PCR, followed by direct sequencing of purified fragments as described (32).

hMLH1 expression analysis. Immunohistochemical analyses were performed and scored on 4-μm sections of formalin-fixed, paraffin-embedded cancer tissue and adjacent normal tissue using a monoclonal antibody against hMLH1, as previously described (7). Two investigators reviewed the immunohistochemical staining profiles independently and discrepancies were reexamined and discussed with a pathologist until consensus was reached. hMLH1 expression status was determined successfully in 721 (98%) of 734 patients.

Food frequency questionnaire. The self-administered questionnaire was a 150-item semiquantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study and also contained questions about body weight and length, smoking status, physical activity, and family history of colorectal cancer. Daily mean nutrient intakes were calculated as the cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986) (33). The questionnaire was validated through comparison with a 9-d diet record (34). Questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case or subcohort status. This was done to minimize observer bias in coding and interpretation of the data.

Folate data were derived from a validated liquid chromatography trienzyme method (35) used to analyze the 125 most important Dutch foods contributing to folate intake (36). Mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (33). Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamins and/or multivitamin supplements was low (7 and 4%, respectively), and folic acid was generally not included in supplements in The Netherlands in the late 1980s. Therefore, folic acid supplement use probably plays a very minor role in our study population, and dietary supplement use was not further accounted for in the analyses. Cases and subcohort members with incomplete or inconsistent dietary data were excluded from analyses (27,34). Hence, 446 colon cancer cases, 160 rectal cancer cases, 76 rectosigmoid cancer cases, and 4343 subcohort members remained.

Statistical analyses. Data analyses were conducted overall, as well as stratified for men and women, and for colon and rectal tumors with and without truncating *APC* mutations, described here as APC^+ tumors and APC^- tumors, respectively. The group of cases without truncating *APC* mutations consisted of cases with tumors containing a wild-type *APC* gene (28%), a missense mutation (29%), or a silent mutation (6%). Because the number of patients with a rectosigmoid tumor was too small for adequate stratified analyses, we included these patients only when assessing associations for all colorectal tumors combined. Furthermore, the rectosigmoid is regarded as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum (32).

The analyses were repeated for tumors with *APC* mutations, excluding those tumors that also contained activating *K-ras* mutations and were hMLH1 deficient. Of 125 truncating *APC* colon tumors, 64 (49%) exclusively contained a truncating *APC* mutation. To investigate the effect of folate on specific point mutations, analyses were conducted for tumors with G:C > A:T mutations in *APC* irrespective of the presence of truncating *APC* mutations or other gene defects.

The intake of dietary folate and other baseline characteristics were evaluated for subcohort members and colon and rectal cancer cases, with or without *APC* mutations, by calculating the mean and SD of the continuous variables, which included folate intake (μg/d), age (y), BMI (kg/m^2), energy (kJ/d), alcohol (g/d), total fat (g/d), fiber (g/d), vitamin C (mg/d), riboflavin (mg/d), vitamin B-6 (mg/d), iron (mg/d) and methionine (mg/d). Distributions of the variables for family history of colorectal cancer (yes/no), smoking status (never/ex/current smoker), and physical activity during leisure time (<30, 30–60, 60–90, >90 min/d) were also calculated. Differences in mean values or distributions of variables between groups of cases with and without truncating *APC* mutations were tested with the Student's *t* test, the Mann-Whitney U test, or the chi-square test where appropriate.

Cox proportional hazards regression models were used to estimate age-adjusted and multivariate-adjusted gender-specific incidence rate ratios (RR) and corresponding 95% CI of colon and rectal cancer for tertiles of folate intake. In addition, associations were estimated for tumors with or without truncating *APC* mutations. Standard errors of the RRs were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (37). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (38). Tests for dose response trends over the different tertiles of folate intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test.

The covariates included in the multivariate analyses were those found to significantly contribute to the multivariate model ($P \leq 0.05$) for colon and/or rectal cancer, or to influence the RR by >10%. This applied to the variables for age, family history of colorectal cancer, BMI, energy, fiber, vitamin C, riboflavin, vitamin B-6, iron and methionine. After excluding subjects with missing information on ≥1 of these covariates, 4200 subcohort members remained for statistical analyses, as well as 399 colon and 129 rectal cancer cases with complete *APC* and *K-ras* mutation analyses, and 394 colon and 128 rectal cancer cases with additional complete hMLH1 expression analyses.

Several factors were previously found to be associated with colorectal cancer risk or to be capable of modifying the association between dietary folate and colorectal cancer risk, i.e., smoking (22,39,40), alcohol (41,42), riboflavin, B-6 (43,44), and iron intake (45). We therefore determined possible effect modification of these factors, as well as for sex, by testing for interaction and by stratified analyses.

All statistical analyses were performed with the STATA statistical software package (Intercooled STATA, version 9.1).

Results

We explored mean dietary intakes and other characteristics measured at baseline for cancer cases and subcohort members. Folate intake among males with APC^+ colon tumors was higher compared with males with APC^- colon tumors (**Table 1**). Furthermore, we observed that intakes of riboflavin and iron were significantly higher among men with APC^+ colon tumors compared with men with APC^- colon tumors. All other characteristics presented in Table 1 did not differ substantially between tumors with and without truncating *APC* mutations.

To further investigate the associations among folate intake, colon cancer risk, and *APC* mutation status, we calculated relative risks for men and women (**Table 2**). Folate intake was not associated with overall colon cancer risk in either men or women. However, it reduced the risk of APC^- colon tumors in men (RR 0.58, CI 0.32–1.05 for the highest vs. the lowest tertile of folate intake; $P_{trend} = 0.06$). In contrast, there was a strong positive association between dietary folate and APC^+ colon tumors in men (RR 2.77, CI 1.29–5.95, $P_{trend} = 0.008$), whereas no association could be observed in women with this type of colon tumor. When calculating associations between folate intake and cancer risk for APC^+ colon tumors without an additional mutated *K-ras* gene and with hMLH1 expression, the positive association appeared even stronger in men (RR 3.99, CI 1.43–11.14, $P_{trend} = 0.007$) but not in women. High folate intake

TABLE 1 Baseline dietary intake and other characteristics of cancer cases and subcohort members from the Netherlands Cohort Study on diet and cancer in 1986[1]

	Men					Women				
		Colon		Rectum			Colon		Rectum	
Characteristic	Subcohort	*APC*$^{-}$ [2]	*APC*$^{+}$ [3]	*APC*$^{-}$	*APC*$^{+}$	Subcohort	*APC*$^{-}$	*APC*$^{+}$	*APC*$^{-}$	*APC*$^{+}$
n	2040	143	70	46	38	2136	131	55	26	19
Folate, *μg/d*	224.9 ± 75.2	215.8 ± 65.6	247.9 ± 67.9*	221.6 ± 52.1	218.1 ± 69.8	198.7 ± 67.2	186.7 ± 69.2	195.2 ± 56.9	218.5 ± 94.8	211.0 ± 56.9
Age, *y*	61.3 ± 4.2	63.0 ± 4.3	62.7 ± 3.9	62.2 ± 4.7	62.4 ± 3.6	61.4 ± 4.3	63.3 ± 3.9	62.7 ± 4.2	62.2 ± 4.0	62.9 ± 3.3
Family history, *% yes*	5.4	11.9	8.6	6.5	15.8	5.7	9.9	12.7	15.4	0
BMI, *kg/m*^{2}	25.0 ± 2.6	25.5 ± 2.9	25.7 ± 2.9	25.2 ± 2.6	24.8 ± 2.7	25.1 ± 3.56	25.4 ± 3.7	25.9 ± 3.2	25.4 ± 3.9	25.7 ± 3.0
Smoking status, %										
Never	12.8	11.9	8.6	8.7	13.2	58.2	61.8	74.6	57.7	73.7
Exsmoker	51.7	65.7	64.3	56.5	52.6	20.6	24.4	16.4	30.8	10.5
Current smoker	35.5	22.4	27.1	34.8	34.2	21.2	13.7	9.1	11.5	15.8
Physical activity,[4] %										
<30 min/d	17.7	9.9	20.3	13.3	15.8	24.3	28.7	33.3	23.1	47.4
30–60 min/d	30.5	35.5	30.4	20.0	36.8	31.3	31.0	27.8	42.3	21.1
60–90 min/d	18.8	22.0	14.5	22.2	13.2	22.7	21.7	20.4	23.1	26.3
>90 min/d	32.0	32.6	34.8	44.4	34.2	21.7	18.6	18.5	11.5	5.3
Dietary factors										
Energy, *kJ/d*	9084 ± 2134	8815 ± 1698	9183 ± 2166	9204 ± 1769	8875 ± 1604	7044 ± 1656	6785 ± 1492	7239 ± 2003	7071 ± 1415	7592 ± 1296
Alcohol,[5] *g/d*	14.9 ± 16.8	15.3 ± 15.9	16.8 ± 18.8	15.2 ± 17.1	18.6 ± 19.4	5.8 ± 9.5	6.2 ± 12.2	3.9 ± 9.2	6.8 ± 7.9	5.8 ± 11.2
Fat, *g/d*	94.3 ± 28.4	92.0 ± 23.5	94.1 ± 28.8	93.0 ± 24.5	89.7 ± 25.4	73.9 ± 22.7	71.5 ± 21.9	77.1 ± 25.8	74.3 ± 22.2	83.5 ± 18.8
Fiber, *g/d*	28.7 ± 8.7	28.9 ± 7.7	30.3 ± 7.9	30.0 ± 7.7	29.1 ± 10.2	25.3 ± 7.0	24.2 ± 7.5	24.8 ± 7.2	24.6 ± 4.0	26.3 ± 5.3
Vitamin C, *mg/d*	98.8 ± 42.9	102.3 ± 42.9	111.6 ± 42.9	113.9 ± 51.9	98.7 ± 48.0	108.6 ± 44.5	101.9 ± 48.8	105.6 ± 43.1	114.8 ± 34.0	117.5 ± 52.2
Riboflavin, *mg/d*	1.58 ± 0.45	1.53 ± 0.39	1.65 ± 0.44*	1.53 ± 0.35	1.49 ± 0.34	1.45 ± 0.41	1.37 ± 0.34	1.46 ± 0.44	1.53 ± 0.48	1.53 ± 0.29
Vitamin B-6, *mg/d*	1.54 ± 0.38	1.54 ± 0.35	1.62 ± 0.37	1.58 ± 0.35	1.46 ± 0.40	1.33 ± 0.32	1.28 ± 0.30	1.37 ± 0.37	1.34 ± 0.27	1.45 ± 0.32
Iron, *mg/d*	13.2 ± 3.3	13.3 ± 3.0	14.5 ± 2.8*	13.6 ± 2.6	13.5 ± 3.3	11.7 ± 2.7	11.3 ± 2.4	11.3 ± 2.5	11.5 ± 2.1	12.1 ± 2.1
Methionine, *mg/d*	1716 ± 417	1694 ± 366	1736 ± 387	1658 ± 298	1598 ± 408	1492 ± 366	1442 ± 304	1537 ± 447	1140 ± 337	1613 ± 290

[1] Values are means ± SD or %, *Different from *APC*$^{-}$, $P < 0.05$.
[2] Tumors without a truncating *APC* mutation.
[3] Tumors with a truncating *APC* mutation.
[4] Information on physical activity was not available for 43 subcohort members, for 6 colon cancer cases, and for 1 rectal cancer case.
[5] Information on alcohol intake was not available for 117 subcohort members, for 4 colon cancer cases, and for 4 rectal cancer cases.

tended to reduce the risk of colon tumors in women harboring G:C > A:T point mutations in the *APC* gene (RR 0.45, CI 0.18–1.14; $P_{trend} = 0.10$). This was not observed in men.

Relative risks were also calculated for tumor sublocalizations within the colon. However, it appeared that folate intake was neither associated with proximal and distal colon cancer risk, nor did these relative risks substantially differ from overall colon cancer risk. Accounting for *APC* mutations in these subgroups did not reveal differential results (data not shown).

Regarding rectal cancer, we observed that intake of dietary folate was neither associated with overall cancer risk nor with rectal cancer, with or without *APC* truncating mutations, in either men or women (see **Table 3**). However, when conducting the analyses for *APC*$^{+}$ tumors without a *K-ras* mutation and with hMHL1 expression, folate tended to be positively associated with rectal cancer risk among men (RR 3.69, CI 0.97–13.98; $P_{trend} = 0.06$).

In addition, we assessed possible associations for all colorectal cancer cases combined for each of the endpoints considered in this study. Folate intake was also positively associated with *APC*$^{+}$ colorectal tumors without a *K-ras* mutation and with hMHL1 expression (RR 1.86, CI 0.97–3.55), again, most pronounced among men (RR 3.40, CI 1.54–7.50), although the interaction with sex was not significant. Significant interactions were observed between folate intake and sex in the associations with overall colon cancer risk ($P = 0.03$), and *APC*$^{+}$ colon tumors ($P = 0.01$). None of the other interactions tested were significant, nor did the remaining stratified analyses reveal any different relative risks compared with the overall groups.

Discussion

In this study, we investigated the associations between folate intake and colorectal carcinoma risk with and without truncating *APC* mutations. We observed that folate intake in the 2nd and highest tertile reduced the risk of *APC*$^{-}$ colon tumors among men, whereas folate intake was positively associated with *APC*$^{+}$ colon tumors in men. This positive association among men was even stronger for *APC*$^{+}$ colon tumors, and appeared in rectal tumors, when tumors with additional aberrations in *K-ras* and with hMLH1 expression were excluded.

To our knowledge, the relation between folate and *APC* mutations was investigated only once before in colorectal adenomas by Diergaarde et al. (9), but, in contrast to our study, no differences were observed between the *APC*$^{-}$ and *APC*$^{+}$ endpoints. Reasons for this discrepancy might be that adenomas were studied instead of carcinomas, and that a case-control design was used with selected cases vs. a case-cohort design with incident cases in our study. Furthermore, in the study of Diergaarde et al. (9), the analyses were not stratified for gender, which may have attenuated potential associations in men. On the other

hand, the ranges of dietary folate intake within the tertiles of intake were comparable to those in our study population. In another study by Diergaarde et al. (10), intake of green leafy vegetables, an important source of dietary folate, was inversely associated with APC^- colon carcinomas. Although the association with folate intake was not calculated separately in that study, the results suggest a similarity with the inverse association between folate and APC^- colon carcinomas in our study. However, the reason why the protective influence of folate is confined to these tumors remains unclear and needs further investigation.

After determining potential interactions, we found that sex significantly modified the effect of folate intake on overall colon cancer risk, as well as on APC^+ colon tumors. For this reason, we presented the data for men and women separately despite the drawbacks of thereby creating smaller subgroups and less-precise estimations. To minimize the potential danger of reporting chance findings, however, we also conducted analyses for all colorectal tumors combined, with and without stratifying for sex. We then observed that folate intake was again positively associated with

TABLE 2 RR and 95% CI for colon cancer patients according to tertiles of folate intake

	Men			Women		
Tertiles of folate intake[1]	N[2]	RR[3]	95% CI	N[2]	RR[3]	95% CI
Colon carcinoma, all tumors						
1	74	1.00		74	1.00	
2	56	0.69	0.46–1.02	64	0.94	0.61–1.44
3	83	0.96	0.61–1.54	48	0.82	0.45–1.49
P-value for linear trend		0.84			0.53	
Colon carcinoma,[4] APC^-						
1	60	1.00		56	1.00	
2	39	0.56	0.35–0.88	42	0.85	0.52–1.39
3	44	0.58	0.32–1.05	33	0.79	0.40–1.54
P-value for linear trend		0.06			0.47	
Colon carcinoma,[5] APC^+						
1	14	1.00		18	1.00	
2	17	1.20	0.56–2.55	22	1.21	0.54–2.70
3	39	2.77	1.29–5.95	15	0.91	0.27–3.06
P-value for linear trend		0.008			0.91	
Colon carcinoma,[6] APC^+, K-ras$^-$, hMLH1$^+$						
1	7	1.00		11	1.00	
2	10	1.58	0.57–4.35	7	0.67	0.18–2.48
3	22	3.99	1.43–11.14	7	0.77	0.13–4.57
P-value for linear trend		0.007			0.75	
Colon carcinoma, G:C > A:T point mutations in *APC*						
1	40	1.00		39	1.00	
2	32	0.66	0.39–1.12	34	0.78	0.41–1.49
3	48	0.86	0.46–1.61	20	0.45	0.18–1.14
P-value for linear trend		0.63			0.10	

[1] Median intakes of folate within tertiles of folate are 162.7, 211.4, and 279.9 μg/d for men and 142.5, 186.8, and 248.0 μg/d for women.
[2] Number of person years among subcohort members for the 1st, 2nd, and 3rd tertile of folate intake is 3243, 3282, and 3293 for men and 3457, 3512, and 3526 for women.
[3] RR adjusted for age, family history, BMI, iron, fiber, energy, riboflavin, vitamin B-6, vitamin C, and methionine.
[4] Colon tumors without a truncating *APC* mutation.
[5] Colon tumors with a truncating *APC* mutation.
[6] Colon tumors with a truncating *APC* mutation, no *K-ras* mutation, and with hMLH1 expression.

TABLE 3 RR and 95% CI for rectal cancer patients according to tertiles of folate intake

	Men			Women		
Tertiles of folate intake[1]	N[2]	RR[3]	95% CI	N[2]	RR[3]	95% CI
Rectal carcinoma, all tumors						
1	26	1.00		10	1.00	
2	32	1.11	0.63–1.97	18	1.71	0.72–4.04
3	26	0.91	0.41–2.01	17	1.54	0.55–4.33
P-value for linear trend		0.82			0.42	
Rectal carcinoma,[4] APC^-						
1	12	1.00		6	1.00	
2	19	1.31	0.62–2.77	10	1.78	0.58–5.49
3	15	0.93	0.31–2.72	10	1.80	0.46–6.98
P-value for linear trend		0.89			0.39	
Rectal carcinoma,[5] APC^+						
1	14	1.00		4	1.00	
2	13	0.95	0.39–2.31	8	1.60	0.41–6.29
3	11	0.92	0.29–2.99	7	1.25	0.25–6.34
P-value for linear trend		0.89			0.82	
Rectal carcinoma,[6] APC^+, K-ras$^-$, hMLH1$^+$						
1	5	1.00		1	1.00	
2	7	1.98	0.63–6.21	4	3.42	0.30–38.89
3	9	3.69	0.97–13.98	3	2.56	0.16–41.13
P-value for linear trend		0.06			0.52	
Rectal carcinoma, G:C > A:T point mutations in *APC*						
1	12	1.00		7	1.00	
2	18	1.34	0.61–2.95	8	1.25	0.37–4.22
3	16	1.15	0.39–3.35	9	1.62	0.39–6.64
P-value for linear trend		0.80			0.50	

[1] Median intakes of folate within tertiles of folate are 162.7, 211.4, and 279.9 μg/d for men, and 142.5, 186.8, and 248.0 μg/d for women.
[2] Number of person years among subcohort members for the 1st, 2nd, and 3rd tertile of folate intake is 3243, 3282 and 3293 for men, and 3457, 3512 and 3526 for women, respectively.
[3] RR adjusted for age, family history, BMI, iron, fiber, energy, riboflavin, vitamin B-6, vitamin C and methionine.
[4] Rectal tumors without a truncating *APC* mutation.
[5] Rectal tumors with a truncating *APC* mutation.
[6] Rectal tumors with a truncating *APC* mutation, no *K-ras* mutation, and with hMLH1 expression.

APC^+ colorectal tumors without a *K-ras* mutation and with hMHL1 expression, and that this effect was only present among men.

Although one could argue that the number of cases in some subgroups should preferably have been higher, we emphasize that the selection of patients in this study was based on the availability of tissue samples with sufficient extracted DNA as well as the completeness of analyses of the *K-ras* and *APC* genes and hMLH1 expression. This led to a reduction of the number of cases that could be included in the analyses. However, a tissue sample, as well as a sufficient amount of DNA, was available for 90% of the patients. Moreover, the overlap of available molecular analyses was high (89%). Finally, it is important to realize that the characteristics of these patients with regard to age, sex, family history of colorectal cancer, smoking behavior, and dietary factors differed neither from the 815 patients initially identified nor from the 734 patients with sufficient tumor DNA. It is therefore unlikely that bias occurred due to the selection of patients.

High folate intake resulted in an increased risk of APC^+ colon tumors among men. However, this was not expected, given the hypothesis that folate prevents DNA damage. Because it is reasonable to assume that aberrations in other key genes in colorectal carcinogenesis may also be associated with folate intake, we included *K-ras* mutation status and hMLH1 expression in our analyses to exclude their potential underlying influence. The observations that *APC* and *K-ras* mutations may be inversely associated with MSI (14–17) and with hMLH1 expression (18,19) are important reasons to do so. *APC* mutated colon tumors, without additional *K-ras* mutation and with hMLH1 expression, were even more positively associated with folate intake in men, and the positive association also appeared for this type of rectal tumor among men. Interestingly, folic acid supplementation might have a cancer-promoting effect in people with already-existing, undiagnosed premalignant or malignant lesions (4,46). *APC* mutations play an important role in both tumor initiation as well as in later stages of colorectal cancer development (47). The development of lesions containing early *APC* mutations is possibly more sensitive to a high folate intake; that is, the protective influence of dietary folate on cancer progression might be decreased after an *APC* mutation has occurred compared with tumors that were initiated otherwise.

Other recent studies confirm that multiple alternative genetic pathways to colorectal cancer may exist, because it was found that only a small number of colorectal tumors (5–6%) harbored mutations in all 3 key genes *K-ras, APC*, and *TP53* (48,49). We observed strong positive associations for tumors containing exclusively *APC* mutations in men only. This may indicate that folate potentially mediates an *APC* mutated pathway in the colon and rectum in men, but that its effect differs between men and women. However, the reasons for these differences are unknown, and clearly, other studies are warranted to confirm or reject this.

The relative risks of tumors harboring G:C > A:T point mutations in *APC* were slightly decreased among women with colon tumors. Decreased activity of the DNA repair gene O^6-*MGMT* by epigenetic hypermethylation might be an indirect mechanism through which folate intake modifies colorectal cancer risk (11). The O^6-MGMT protein removes adducts from the O^6 position of guanine in DNA (12), which in turn prevents G:C > A:T point mutations. Previously, we reported that O^6-*MGMT* promoter hypermethylation may partly be due to a low folate status (50). It has also been reported that that O^6-*MGMT* hypermethylation is associated with G:C > A:T mutations in *K-ras* and *TP53* (12,13,27). However, others did not observe an association between O^6-*MGMT* expression and G:C > A:T mutations in the *APC* gene alone (51). Further research is therefore needed to investigate whether *APC* mutations might occur via decreased O^6-*MGMT* functionality.

A potential protective effect of folate intake on colorectal cancer risk has been demonstrated only in 10 of 23 epidemiological studies investigating this association (2). In addition, we observed higher risks of colorectal cancer in some subgroups even though intakes of dietary folate in our study population were relatively low compared with some other Western countries. This raises the question as to whether high folate intake should be a reason for concern in terms of colorectal cancer risk, particularly in view of differences in recommended dietary intakes between countries and the mandatory fortification of cereals with folic acid in the United States and Canada. It is therefore of crucial importance to gain more insight into the role that dietary folate plays in colorectal carcinogenesis with genetic aberrations like truncating *APC* mutations in future studies.

In conclusion, the results of this study indicate that an inverse association between relatively high folate consumption and carcinogenesis is limited to APC^- colon tumors, especially in men. Combined with the higher relative risks of APC^+ colon and rectal tumors without additional aberrations in 2 other key genes involved in colorectal cancer in men, these findings may indicate that folate intake exerts an effect on specific genetic pathways, i.e., an *APC* mutated pathway. Tumors that arise through distinct genetic pathways on the basis of specific genetic aberrations, such as truncating *APC* mutations, possibly have a unique etiology, and folate may have a different effect on these pathways in men and women.

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